SHORT COMMUNICATIONS

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Crystallization and preliminary X-ray analysis of the river buffalo (*Bubalus bubalis L.*) BB phenotype carbonmonoxyhaemoglobin.

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Abstract

Ruminant haemoglobin (Hb) extracted from river buffalo (*Bubalus bubalis*) has been purified and crystallized. Two different Hb forms of the phenotype BB gave isomorphous crystals which diffracted to 2.8 Å resolution and were not sensitive to radiation damage. Crystals of CO Hb have space group $P2_12_12_1$ with unit-cell parameters $a = 54.8$, $b = 64.0$, $c = 158.6$ Å, and contain one Hb molecule per asymmetric unit.

Introduction

Bovine Hb* has been widely investigated, because of its different functional properties, such as a low oxygen affinity even in the absence of organic phosphates, in comparison with human Hb (Perutz & Imai, 1980). Among various bovine Hb's extracted from different species, that extracted from river buffalo (*Bubalus bubalis*) shows different behaviour compared with other ruminant Hb's. Recent studies on this Hb have provided evidence of a high polymorphism. Among the six phenotypes detected so far, the most common one, BB, consists of two principal Hb components, named Hb2 and Hb4, which differ only in their α-chains (Di Luccia, Iannibelli, Addato, Masala, Manca & Ferrara, 1991). The sequences exhibit a high similarity with those of other *Bovidae*, *viz.* the similarity of phenotype AA Hb is 97% compared with that of *Bos taurus* (Ferranti *et al.*, 1992). As expected, Hb from the BB phenotype is also characterized by a low oxygen affinity as well as by a reduced sensitivity to organic phosphates (*viz.* GriP2) (Giardina *et al.*, 1992). On the other hand, the effect of temperature on oxygen binding is significantly higher than that displayed by other ruminant Hb species.

In recent years, the *intrinsic* oxygen affinity of human and bovine Hb was considered to be comparable, taking into account that bovine Hb is more sensitive to chloride than human Hb (Fronticelli, Bucci & Orth, 1984). On this basis, oxygen binding was proposed to be regulated by chloride anions and several specific chloride-binding sites were postulated (Fronticelli, 1990; Ueno, Pospischil & Manning, 1989; Ueno & Manning, 1992). Conversely, the very recent crystal structure determination of bovine deoxyhaemoglobin has provided evidence of the absence of the predicted chloride-binding sites. As a result, a novel allosteric mechanism was proposed (Fermi, Perutz, Poyart, Pagnier & Kister, 1993).

In this context, the structural knowledge of closely related Hb's, in the liganded and unliganded states, appears to be of interest. The determination of the crystal structure of CO Hb, from the BB phenotype of the river buffalo, may provide the structural details of the *R* state of bovine Hb as well as the molecular basis for its peculiar behaviour. The present report describes the purification, the crystallization conditions and a preliminary X-ray analysis of the carbonmonoxy derivative of Hb2 and Hb4 components.

Materials and methods

Purification of Hb from the BB phenotype

Blood samples from river buffalo were collected and treated following standard procedures in our laboratory, as described by Di Luccia *et al.* (1991). Two different procedures have been used to separate the Hb components from a lysate of BB phenotype. In one case, FPLC (Pharmacia, Sweden) was performed using a strong anionic exchange Neobar AQ column (Dynochrom, Norway). A 0–30 m*M* NaCl linear gradient in 20 m*M* Tris–HCl, pH 8.0, was applied for 30 min. Hb4 and Hb2 were eluted at NaCl concentrations of 20 and 27 m*M*, respectively.

A lysate of Hb BB phenotype was also purified by preparative IEF in IPG, ranging in pH from 6.8 to 7.8. A preparative polyacrylamide gel was prepared according to the manufacturer's instructions (Application Note 323, Pharmacia/LKB, Uppsala, Sweden). Bands corresponding to Hb2 and Hb4 were cut out, and the strip gels were equilibrated overnight and then electroeluted in electrode buffer (25 m*M* Tris, 192 m*M* Gly, pH 8.3) using a Model 422 Electro-eluter (Bio-rad, California).

Crystallization and X-ray measurements

Several crystallization trials have been performed by various methods (hanging drop, dialysis, microbatch) (Weber, 1991). At two different temperatures (277 and 293 K), the effect of several precipitant agents (PEG 8000, ammonium sulfate, potassium phosphate and MPD) has been tested, varying pH from 6.5 to 8.3 and protein concentration in the range 6–20 mg ml^{-1}. All solutions

* Abbreviations: Hb, haemoglobin; GriP2, 2,3-bisphosphoglycerate; PEG, polyethylene glycol; MPD, 2-methyl-2,4-pentane diol; FPLC, fast protein liquid chromatography; IEF, isoelectric focusing; IPG, immobilized pH gradient.

were saturated with CO, before use. Preliminary screenings performed on an IMPAX Automatic Protein Crystallization System (Douglas Instruments Ltd, UK) gave useful information on crystallization conditions. Then crystals were grown by a microbatch procedure: 20–30 μl of solutions containing either Hb4 (12.5–20.0 mg ml^{-1}), sodium potassium phosphate (2.65–2.85 *M*) pH 6.4–6.7, or Hb2 (10.0–12.0 mg ml^{-1}), sodium potassium phosphate (2.70–2.85 *M*) pH 6.7, were incubated in 1.5 mm diameter glass capillaries at 277 K. Within a few weeks, a precipitate was observed in all samples and prismatic crystals, to a maximum size of 0.5 × 0.5 × 0.4 mm, were formed only in a few parallel tests (20–50%), indicating a lack of reproducibility. Crystals of each Hb species were washed, then dissolved and tested by analytical IEF. Only the expected band was detected.

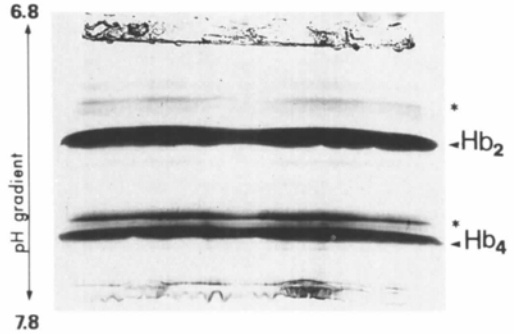

Fig. 1. Preparative isoelectric focusing in the immobilized pH gradient (6.8–7.8) of lysates from river buffalo BB phenotype. Asterisks denote bands not identified.

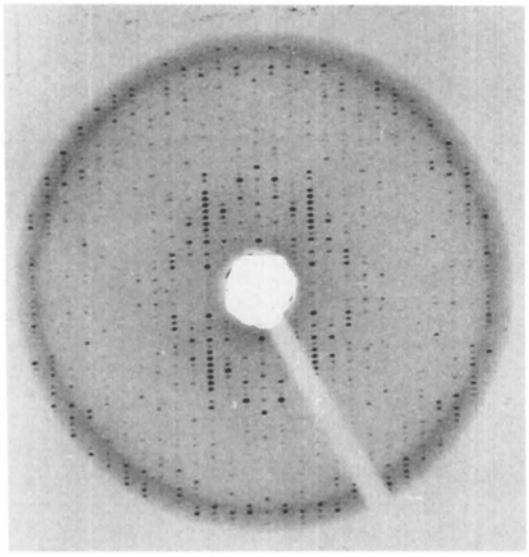

Fig. 2. An 11° precession photograph (*h0l*) of the Hb4 haemoglobin crystals. Crystal-to-film distance is 6 cm.

Precession photographs were obtained using an Elliot GX-6 rotating-anode source operating at 40 kV and 40 mA (Cu $K\alpha$) at room temperature.

Results and discussion

Hb2 and Hb4 purification

The FPLC profile, using an anion-exchange column, showed two peaks which were contaminated by components with a higher isoelectric point.

On the other hand, the IPG technique allowed separation of Hb2 and Hb4 bands from the other Hb components without distortion of the pH gradient. Therefore, the two Hb forms were eluted from strip gels with high purity. In Fig. 1 is shown the preparative IEF profile of lysates from river buffalo BB phenotype. Beside the two principal components, Hb2 and Hb4, additional bands, not yet identified, were detected.

Crystal data

A systematic search for crystallization conditions led us to obtain isomorphous single crystals of both carbonmonoxy principal Hb components of the river buffalo BB phenotype. X-ray precession photographs of zero-level reciprocal lattice sections showed orthorhombic symmetry. Systematic absences are consistent with space group $P2_12_12_1$. The diffraction pattern extends to at least 2.8 Å resolution and few reflections are observed up to 2.5 Å. Crystals were not sensitive to radiation damage. The diffracting quality is not affected by different purification procedures. Fig. 2 shows a 11° precession photograph of the *h0l* reciprocal lattice section. The unit-cell parameters are $a = 54.8$, $b = 64.0$, $c = 158.6$ Å, $V = 5.57 \times 10^5$ $Å^3$, assuming that the asymmetric unit contains one Hb tetramer, the calculated V_m is 2.16 $Å^3$ Da^{-1}. This value compares well with those reported for other haemoglobins (Matthews, 1968).

Hopefully, structural information derived from the determination of the crystal structure of the river buffalo carbonmonoxy Hb, in conjunction with the available structural information of bovine deoxy Hb (Fermi *et al.*, 1993), could contribute to the knowledge of allosteric regulation of haemoglobin in mammals.

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